The post-embryonic development of *Amphiprion perideraion* reveals a decoupling between morphological and pigmentation changes

by

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Abstract. – During coral reef fish life cycle, the young pelagic larvae colonize the reef. During such important ecological transition, larvae undergo metamorphosis consisting on important morphological, colour, physiological and behavioural changes. We investigated the post-embryonic development of *Amphiprion perideraion* Bleeker, 1855 larvae raised in captivity from 1 day post-hatching (dph) until 20 dph. To do so, we characterized larval growth, morphological changes and pigmentation ontogenesis. Interestingly, whereas most of the morphological changes are done from 3 dph to 13 dph, adult coloration appears quite late at 11 dph. This suggests a decoupling between morphological changes and pigmentation changes that appear linked to the metamorphic process.

Résumé. – Le développement post-embryonnaire d'*Amphiprion perideraion* montre un découplage entre les changements morphologiques et pigmentaires.

Au cours du cycle de vie des poissons coralliens, les jeunes larves pélagiques colonisent les récifs coralliens. Au cours de cette transition écologique, les jeunes larves subissent une métamorphose consistant à de nombreux changements morphologiques, physiologiques, comportementaux et de coloration. Dans cet article, nous avons caractérisé le développement post-embryonnaire du poisson corallien *Amphiprion perideraion* Bleeker, 1855 élevé en captivité du jour 1 jusqu'au jour 20 après l'éclosion. Au cours de sa métamorphose, la jeune larve d'*A. perideraion* subit la plupart de ses changements morphologiques entre les jours 3 à 13 après l'éclosion tandis que l'apparition de la coloration adulte commence à partir du jour 11 après l'éclosion. Cela suggère un découplage entre les changements morphologiques et les changements de pigmentation au cours de la métamorphose.

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Coral reef fishes have a complex life cycle that involves ontogenetic changes in morphology, physiology, coloration and behaviour as their pelagic larval stages colonize benthic habitats. The term 'metamorphosis' is used to describe these changes that occur as a larva takes on its juvenile form, which most often coincides with its settlement to the reef (McCormick *et al*., 2002). Recent data suggest that this larval settlement period corresponds to a metamorphosis regulated by a thyroid hormone very similar to the one known in flatfish or frog (Holzer *et al*., 2017; Laudet, 2011).

Clownfishes (*Amphiprion* and *Premnas* species) are well known coral reef fishes famous for their mutualism with sea anemones (Colleye *et al*., 2016). There are in total 30 species composing this tribe (Amphiprionini; Cooper and Santini, 2016) within the Pomacentridae (even if the species status of *A. thiellei* Burgess, 1981 and *A. leucokranos* Allen, 1973 is still debated as they seem to represent natural hybrid). Their colour pattern consists in white vertical bars and/or horizontal stripes on an orange to brown body. Their life cycle includes a relatively short dispersive planktonic larval phase in the open ocean (Berumen *et al*., 2010), followed by the settlement of the juveniles into sea anemones where they live in a social group composed of a dominant breeding pair and a varying number of sexually immature juveniles (Colleye *et al*., 2016).

During the transition between the open ocean and the reef settlement, the larvae undergo a metamorphosis that is essential for their survival and thus for the maintenance of the population (Feeney and Brooker, 2017). Experiments show that *Amphiprion ocellaris* Cuvier, 1830 and *A. frenatus* Brevoort, 1856 acquire their adult vertical white bars during metamorphosis (Yasir and Qin, 2007; Putra *et al*., 2012). The objective of the present study was to document the ontogeny of an *Amphiprion* species that exhibits a white horizontal stripe (*i.e.* a different pigmentation pattern compared to *A. ocellaris* and *A. frenatus*). To do so, we studied the early life of *A. perideraion* having a vertical white head bar and a horizontal dorsal white stripe from the top of the head to the beginning of the caudal fin. More precisely, we

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characterized growth, morphology and pigmentation from post-hatching to juvenile.

MATERIAL AND METHODS

Breeding and larval rearing techniques

A breeding couple of *A. perideraion* was maintained at 27°C in a 60 l aquarium. Breeding pairs laid egg clutches on the underside of a terracotta pot placed in the aquarium. On the night of hatching (10 days post laying at 27°C), egg clutches were transferred from the parental aquarium to a 30 l larval rearing aquarium. Larvae were fed with rotifers (*Brachionus plicatilis*) to a final concentration of 10 individuals per ml), three times a day for the first seven days. Then *Artemia nauplii* was added to the diet, and the ratio of *Artemia nauplii* to rotifers was increased each day until to raise five individuals of *Artemia nauplii* per ml from day 7.

Characterization of growth, morphological and pigmentation changes

Every day, at least two larvae were euthanised using 400 mg/L of MS-222. Then, they were imaged using a Zeiss stereomicroscope (V20 discovery-Plan S objective 1.0x) equipped with a camera (Axiocam 105). Images were then used to follow pigmentation, morphological (fin development) and growth changes. For pigmentation, we did a qualitative description of the main colour changes such as the apparition of the white horizontal stripe and of the vertical bar as well as the change of the trunk coloration from yellow to orange. Measurements were focused on total length (TL): from the tip of the lower jaw to the posterior margin of caudal fin; standard length (SL): from the tip of the snout to the tip of the notochord for larval stage and to the base of the caudal fin (posterior margin of the hypural plate) for the post flexion specimen; head length (HL): from the snout to the extremity of the posterior edge of the opercle; head depth (HD); body depth (BD): from the anus to the upper limit of the myomore excluding dorsal fin hold area (Fig. 1A) (Önsoy *et al*., 2011).

Allometric growth

We tested the null hypothesis of an isometric growth for each studied morphological traits (*i.e*. HL, BD and HD) by using the equation of Huxley $\log y = \log b + a \log x$ (Klingenberg, 1998). If the coefficient $a = 1$, the growth of the trait is isometric. If the coefficient differs from 1, the growth is allometric: the allometry is positive when $a > 1$ and is negative when $a < 1$ (Klingenberg, 1998).

RESULTS

At 1 day post-hatching (dph), larvae measure 4.42 ± 0.21 mm TL (Mean ± SD; 3 individuals). During their post-embryonic development, the growth of the larvae is relatively stable increasing with a mean rate of 0.35 mm/ day at 27°C (Fig. 1A, B). The allometry of HL growth is negative (Fig. 1C, $a = 0.82$) whereas growth of BD and HD show positive allometry (Fig. 1C, $a = 1.6$ and 1.13, respectively). This means that depth of the body and of the head become proportionally bigger during ontogeny.

Morphological changes begin at 3 dph with the start of notochord flexion (Fig. 1D, E, 3 dph). At that time, the caudal fin begins to differentiate, and soft rays form (Fig. 1E, 3 dph). At 4 dph, soft rays of the dorsal and anal fins appear (Fig. 1F, G, 4 dph). Formation of caudal fin segment starts at 6 dph (Fig. 1E, 6 dph) whereas the dorsal spines appear at 8 dph (Fig. 1F, 8 dph). The same day, the pelvic fin forms (Fig. 1H, 8 dph) and the spines of the anal fin appear one day later (Fig. 1G, 9 dph red arrowhead). Finally, dorsal fin segments start to be visible on stereomicroscope images at 10 dph (Fig. 1F, 10 dph), whereas the ones of the anal fins appear at 13 dph (Fig. 1G, 13 dph).

In vertebrates at least eight chromatophores exist, characterized by the composition of their pigment, hence their colour (Schartl *et al*., 2016). Among these chromatophores, the most common ones in fishes are the xantophores which give the orange colour, whereas the black are named melanophores and the white cells can be either iridophores or leucophores (Takeuchi, 1976; Djurdjevič *et al*., 2015). The early post-hatching *A. perideraion* larva is characterized by a transparent body, covered by two horizontal stripes of stellar and very dark melanophores. It is not obvious whether xantophores are already present at 1 dph (Fig. 1D, 1 dph). From 3 dph to 10 dph, the orange coloration increases across the body, and the two melanophores horizontal stripes present at 1 dph remain (Fig. 1D, I, 10 dph). Interestingly, the pigmentation of the larvae changes quite abruptly at 11 dph, and the stellar melanophores and xantophores contract leading to a pale orange coloration of the body (Fig. 1I, compare 10 dph to 11 dph, trunk). At the same time, some very pale grey melanophores are visible forming the future horizontal dorsal stripe whereas only few very dark black melanophores and orange xantophores remain (Fig. 1I, 11 dph trunk under the dorsal fin). The white head vertical bar, surrounded by melanophores, appears at 15 dph (Fig. 1I, 15 dph). It exists two types of pigment which can give the white colour: iridophores and leucophores (Takeuchi, 1976). However, here we could not distinguish what is the chromatophore type. Further experiments need to be done to answer this question.

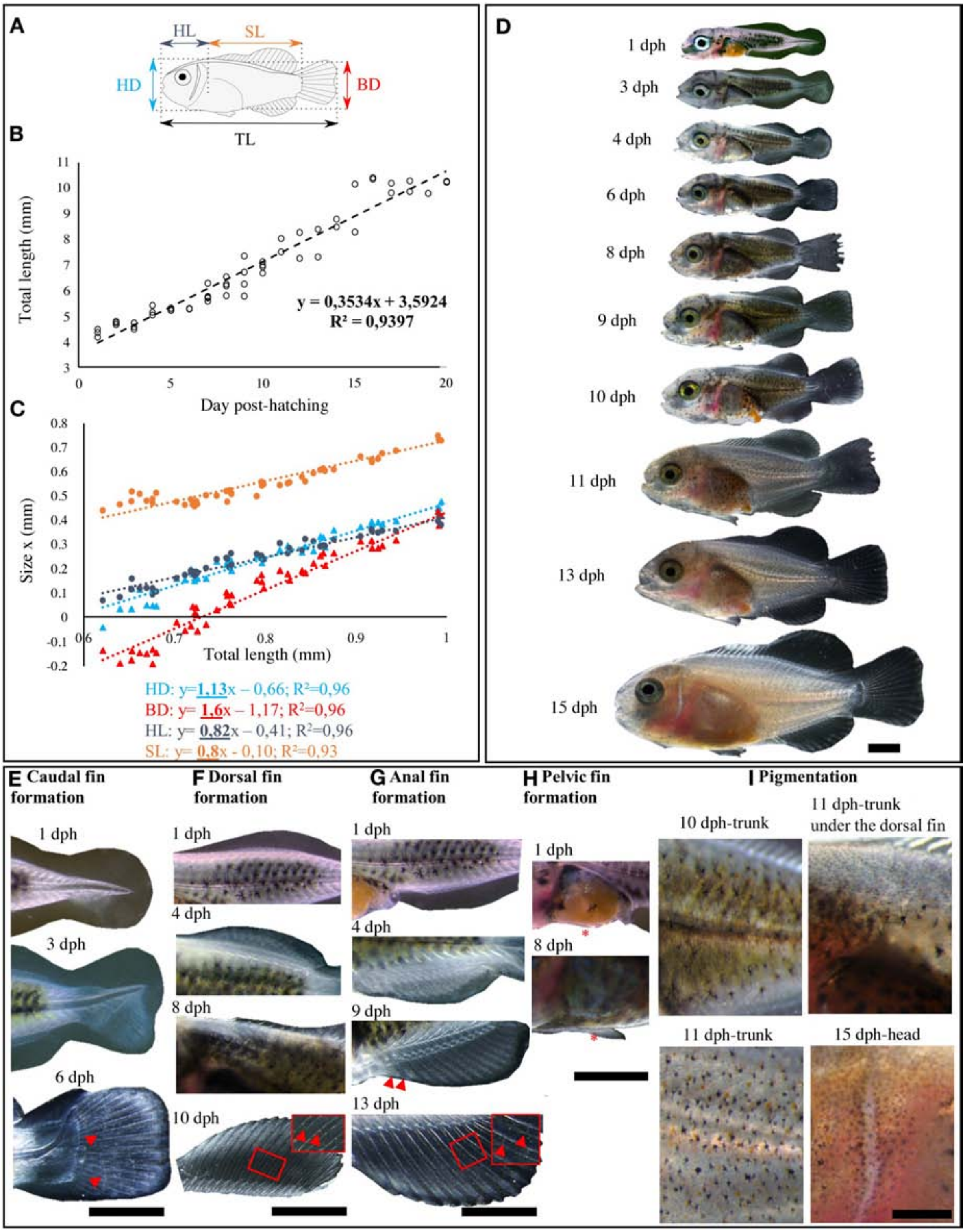

Figure 1. – *Amphiprion perideraion* development from hatching to 20 dph. Graph showing the quantitative measurement of individual larvae from hatching to 20 dph, therefore providing an indication on individual variation. **A**: Morphological characters measured. HD, head depth; HL, head length; SL, standard length; BD, body depth (Allen, 1974; Önsoy *et al*., 2011). **B**: Graph showing the significant relationship among TL to larvae age. **C**: Allometric growth equation between TL and SL (orange), BD (red), HD (turquoise), and HL (blue) in larvae. Note that the allometric growth is negative for HL and positive for BD and HD. **D**: Stereomicroscope images of larvae from 1 dph to 15 dph. **E-I**: Higher magnification of caudal (E), dorsal (F), anal (G) and pelvic (H) fins formation and pigmentation ontogenesis (I). Scale bars = 1 mm.

DISCUSSION

We showed here that the development of the clownfish larvae involves allometric growth as well as morphological and colour pattern changes. The anterior part of *Amphiprion perideraion* larvae becomes proportionally higher during ontogeny. Interestingly, such allometric growth during coral reef settlement is also observed in damselfishes and was shown to be directly linked to an adaptation to their new benthic environments when entering into the reef (Frédérich *et al*., 2008; Frédérich and Vandewalle, 2011). Moreover, we characterized the sequence of morphological changes: with primary formation of caudal fin, soft rays of dorsal fin, anal fin as well as anal and dorsal spines. Such sequence of morphological changes in *A. perideraion* is very similar to what we and others observed in *A. frenatus* and *A. ocellaris* (Yasir and Qin, 2007; Putra *et al*., 2012). However, the rate of development may vary depending on the water temperature and density of larvae (Parichy *et al*., 2009). So to rigorously characterize *A. perideraion* post-embryonic development it would be preferable to account for criteria for staging looking at precise morphological changes rather than using days of development since this may change according to the conditions (NR, PS, DL and VL, in prep.). Such study would be useful to understand the probably more complex post-embryonic development of larva *in situ*.

In vertebrates, including teleost fishes, thyroid hormones and their receptors trigger and coordinate metamorphosis. This is the case in *Xenopus* (Brown and Cai, 2007), flatfish (Solbakken *et al*., 1999) or the coral reef fish *Acanthurus triostegus* (Linnaeus, 1758) (Holzer *et al*., 2017). Intriguingly, in *Amphiprion perideraion,* the formation of the adult pigmentation occurred quite late (11 dph to 15 dph) compared to the changes observed in morphology which occur earlier (from 3 dph to 13 dph). This decoupling between the timing of morphological changes and the timing of adult pigmentation formation suggests that it is controlled by a complex molecular process. Future studies should investigate whether both morphological and pigmentation changes are controlled by thyroid hormones, but also determine the molecular nature of the competence of tissues to differentially respond at a specific time to these or other hormones.

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